LACCASE ACTIVITY ENHANCERS FOR PULP BLEACHING

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/318,290, filed on September 10, 2001.

FIELD OF THE INVENTION

The present invention relates to the enhancement of enzyme activity and the activation of enzymes. More specifically, the present invention relates to mediators, enhancing agents, or activating agents that are useful in enhancing the activity of enzymes having laccase activity, especially in the field of pulp bleaching.

BACKGROUND OF THE INVENTION

Paper pulp is typically processed from wood through the Kraft (and other) processes. The process produces a pulp with a dark brown color, mostly due to the presence of lignin and lignin derivatives. For many applications, the lignin has to be removed by a process known in the art as "delignification" or "bleaching." This is typically done commercially in several stages in pulp mills, wherein lignin is first oxidized and then removed from the pulp. Currently, bleaching of Kraft pulp uses a large amount of chlorine or chlorine-containing compounds. The byproducts of such processes may include chlorinated compounds that are undesirable.

Recently, several research groups have been working with enzymes to biologically bleach pulp, referred to as "bio-bleaching". An enzyme group that has received particular attention is the laccase family of enzymes, which are copper-containing enzymes that are known to be good oxidizing agents in the presence of oxygen. Laccases are found in microbes, fungi, and higher organisms.

Laccase enzymes are used for many other applications, including treatment of pulp waste water, de-inking, industrial color removal, bleach for laundry detergents, oral care teeth whiteners, and as catalysts or facilitators for polymerization and oxidation reactions. For many

applications, the oxidizing efficiency of a laccase can be improved or activated through the use of a mediator, also known as an enhancing agent or activator. Systems that include a laccase and a mediator are known in the art as laccase-mediator systems (LMS).

There are several known mediators for use in a laccase-mediator system. These include HBT (1-hydroxybenzotriazole), ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfinic acid)], NHA (N-hydroxyacetinilide), NHAA (N-acetyl-N-phenylhydroxylamine), HBTO (3-hydroxy-1,2,3-benzotriazin-4(3H)-one), and VIO (violuric acid). In addition, there are several compounds containing NH-OH or N-O that have been found to be useful as mediators.

Functional groups and substituents of the above mediators have large effects on the LMS efficiency. Even within the same class of compounds, a substituent of a mediator can change the specificity of a laccase towards a specific substrate, such as pulp lignin, lignin derivatives or other fine chemicals, thereby increasing or decreasing mediator efficiency greatly. In addition, a mediator may be effective for one particular application but unsuitable for another application. Thus, there is a need to discover efficient mediators for specific applications. One such application is the bleaching of pulp, wherein it is also important that the mediators are not unduly expensive or hazardous. Thus, there is a need to identify additional mediators that activate laccase and/or enhance the activity of enzymes that exhibit laccase activity, particularly for pulp bleaching.

SUMMARY OF THE INVENTION

The invention provides a composition comprising an enzyme exhibiting laccase activity and an enzyme enhancing agent (also referred to as a mediator). The enzyme enhancing agent is selected from 2-thiouracil, sodium dimethyldithio carbonate hydrate, N-benzylidene-benzylamine, melamine, anthracene, dicyandiamide, sulfanilic acid, sulfanilamide,urea, salicylic acid, 3,4,5-trihydroxy-benzoic acid, ferric chloride, potassium ferricyanide, ascorbic acid, Zincon (o-[2-[alpha(2-hydroxy-5-sulfophenylazo)benzylidene]hydrazino]benzoic acid), diisopropanolamine, adenosine triphosphate, guanidine, cyanuric acid, Thiazol Yellow G,

nicotinic acid, Metanil Yellow, hardwood black liquor, softwood black liquor, methanesulfonic acid, metanilic acid, sulfamide, 3-pyridine sulfonic acid, benzofuroxan, t-butyl hydroperoxide, pyruvic acid, imidazole, N-acetylcytosine, and phenol.

The invention also provides a process for oxidizing a substrate that comprises treating the substrate with a composition comprising an enzyme exhibiting laccase activity and an enzyme enhancing agent.

The invention further provides a process for bleaching a lignin-containing material that comprises treating the material with an enzyme exhibiting laccase activity and an enzyme enhancing agent. In one embodiment of the invention, the material is a wood pulp. In another embodiment of the invention, the material is a wood pulp that is a raw material used to form a polysaccharide or a cellulose derivative.

In addition, the invention provides a process for enhancing the activity of an enzyme exhibiting laccase activity that comprises adding an enzyme enhancing agent to the enzyme.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides newly identified enzyme enhancing agents (also referred to as mediators) that enhance the activity of enzymes exhibiting laccase activity.

The mediators of this invention are capable of enhancing the activities of laccase and laccase-related enzymes, i.e., enzymes exhibiting laccase activity. The enzymes exhibiting laccase enzyme activity include the laccase enzymes of enzyme classification EC 1.10.3.2, the catechol oxidase enzymes of enzyme classification EC 1.10.3.1, the monophenol monooxygenase enzymes of enzyme classification EC 1.14.99.1, the bilirubin oxidase enzymes of enzyme classification EC 1.3.3.5, and the ascorbate oxidase enzymes of enzyme classification

EC 1.10.3.3. The EC (Enzyme Commission) number is based upon the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB).

The enzymes exhibiting laccase enzyme activity may be derived from microbial, fungal, or other sources. These enzymes can also be produced by recombinant methods that are well known to those skilled in the art, such as cultivating a host cell transformed with a recombinant DNA vector that includes a DNA sequence encoding a laccase (and, optionally, DNA sequences that permit the expression of the laccase DNA sequence) in a culture medium under conditions that permit the expression of the laccase, and recovering the enzyme from the culture. The composition of the invention can further include a hydrolase, such as xylanase.

The enzyme enhancing agents of the invention are selected from 2-thiouracil, sodium dimethyldithio carbonate hydrate, N-benzylidene-benzylamine, melamine, anthracene, dicyandiamide, sulfanilic acid, sulfanilamide,urea, salicylic acid, 3,4,5-trihydroxy-benzoic acid, ferric chloride, potassium ferricyanide, ascorbic acid, Zincon (o-[2-[alpha(2-hydroxy-5-sulfophenylazo)benzylidene]hydrazino]benzoic acid), diisopropanolamine, adenosine triphosphate, guanidine, cyanuric acid, Thiazol Yellow G, nicotinic acid, Metanil Yellow, hardwood black liquor, softwood black liquor, methanesulfonic acid, metanilic acid, sulfamide, 3-pyridine sulfonic acid, benzofuroxan, t-butyl hydroperoxide, pyruvic acid, imidazole, N-acetylcytosine, and phenol.

These enzyme enhancing agents of the invention can be classified into seven categories:

Group 1. Specific compounds containing nitrogen:

a. guanidine
b. cyanuric acid
c. melamine
d. dicyandiamide
e. urea

f. nicotinic acid

g. N-benzylidene-benzylamine

h. Metanil Yellow

i. Zincon

j. benzofuroxan

k. imidazole

l. N-acetyl cytosine

m. Thiazol Yellow G

Group 2. Ascorbic acid and substituted ascorbic acid

Group 3. Salicylic acid and 3,4,5-trihydroxybenzoic acid

Group 4. Materials from pulp processing

a. hardwood black liquor

b. softwood black liquor

Group 5. Specific compounds containing sulfur:

a. sulfanilamide

b. methanesulfonic acid

c. sulfanilic acid

d. 2-thiouracil

e. metanilic acid

f. sulfamide

g. 3-pyridine sulfonic acid

h. sodium dimethyldithio carbonate hydrate

Group 6. Specific organic compounds:

a. anthracene

b. t-butyl hydroperoxide

c. pyruvic acid

d. diisopropanolamine

e. adenosine triphosphate

f. phenol (conditional)

Group 7. Specific inorganic compounds:

a. ferric chloride

b. potassium ferricyanide

The compounds listed in the above seven groups, their related structures, and structural analogs are all potential mediators for enzyme(s) that exhibit laccase activity.

Another aspect of the invention provides a process for oxidizing a substrate that comprises treating the substrate with a composition comprising an enzyme exhibiting laccase activity and an enzyme enhancing agent. The enzyme enhancing agent can be selected from one or the above described enzyme enhancing agents.

The enhancing agent may be present in concentrations of from about 0.01 micromolar to about 1000 micromolar, more preferably from about 0.1 micromolar to about 250 micromolar and most preferably from about 0.5 to about 100 micromolar.

The enzyme is used in amounts of from about 0.1 to 400 units (defined in Examples using ABTS as substrate) for 1 g dry pulp, more preferably from 1 to 200 units and even more preferably from about 10 to 100 units and most preferably from 20 to 50 units.

The process of the invention can further include the step of adding an oxidizing agent, such as at least one of air, oxygen, and hydrogen peroxide.

One embodiment of the invention provides a process for bleaching a lignin-containing material that comprises treating the material with an enzyme exhibiting laccase activity and an enzyme enhancing agent. In this embodiment of the invention, the enhancing agent may be present in an amount of from about 0.1% to about 15% based on the weight of the dry lignin containing material, more preferably from about 0.1% to about 10% and even more preferably from about 0.5% to about 5% and most preferably from about 1% to about 4 %. One example of a lignin containing material is wood pulp. The process for bleaching a lignin-containing material

can further include the step of adding an oxidizing agent, such as at least one of air, oxygen, and hydrogen peroxide.

The mediators of the invention can be used, for example, for pulp bleaching. Laccase itself can bleach pulp only to a limited extent. The use of the mediators as disclosed herein enhances the activity of laccase in pulp bleaching. In this context, delignification and bleaching efficiency is defined by the "Kappa Number."

EXAMPLES

Delignification of a softwood Kraft pulp using a laccase-mediator system in the presence of a mediator.

The specific activity was determined using ABTS (0.5 mM) as substrate. One unit of activity is equal to the umol of the oxidized product from ABTS per min per mg protein at pH 6.0 at 23 °C. The extinction coefficient of the oxidized ABTS is: ε(max) at 420 nm=36,000M-1cm-1.)

Alternatively, the activity of laccase (NS51003) was determined using syringaldazine as substrate. In this case, one unit of activity is equal to the change of 0.001 UV absorbance at A530nm per minute per ug protein in 2 ml of 100 mM, pH 5.5 potassium phosphate buffer, and 0.5 ml of 0.25 mM syringaldazine in methanol at 23 °C.

In the following example, a softwood Kraft pulp was delignified using a laccase-mediator system. The Kraft process is well known to those skilled in the art and is described, for example, by Swen A. Rydholm in "Pulping Process", Interscience Publishers, New York, NY (1965), p. 576.

A softwood Kraft pulp, Kappa number 31.0, was treated with a laccase (NS51003, Novozymes A/S, Denmark) under the following conditions:

Enzyme dosage	45 units /g pulp
pH	5.5
Temperature	50 °C
Reaction time	16 hours
Pulp consistency	2%

The dried pulp was added to 80 ml of 50 mM phosphate, pH 5.5, and disintegrated in a blender. The pulp was then transferred to a 500 ml conical flask, and the blender was washed with 20 ml of the same buffer. The washed buffer and the pulp were combined. The mediator was added at 1-4% (w/w, based on the dry pulp) (Dosage) followed by the addition of the laccase. The pH of the pulp mixture was adjusted to 5.5 if needed. The flask was covered with an aluminum foil with holes punched through and incubated at 50 °C for 16 hrs on a rotary shaker at 200 rpm.

After the enzymatic treatment, the pulp mixture was filtered through a Buchner funnel and the pulp was washed with water. The pulp from the control experiment was treated at the same pH and temperature as described above in the absence of mediators.

The washed pulp was then treated with an alkaline solution under the following conditions:

Pulp	2 g
Water	200 ml
NaOH	240 mg
H_2O_2 (30%)	400 ul
Temperature	70 °C
Reaction time	3 hours

The filtered pulp was repulped in the alkaline solution and incubated at 70 °C for 3 hrs. The pH of the pulp mixture should be between 11.7-12.00 during the entire treatment. After the

treatment, the pulp mixture was filtered through a Buchner funnel, and the pulp was washed with water extensively, and then dried in a hood overnight.

The delignification of the pulp was measured as the change in Kappa number according to TAPPI method T236 cm-85. Briefly, a known mass of paper pulp (containing lignin) was reacted with an excess of potassium permanganate in acid solution for a specified period of time to oxidize the lignin. After the reaction, the residual permanganate was determined by titration. The Kappa number was defined as the volume (ml) of 0.1N potassium permanganate consumed by 1 g of moisture-free pulp in 0.5N sulfuric acid after a ten minute reaction time at 25 °C under conditions such that one-half of the permanganate remained unreacted. A linear relationship with the lignin content of the pulp and the measurement of the Kappa number had already been done on samples as low as 300 mg of pulp. The results are shown below.

Mediator(s)	*Dosage (%)*	*Laccase (%)*	*Kappa # (No Mediator)*	*Kappa # (Mediator)*	*Δ Kappa #*
2-Thiouracil	4	0.2	29.3	27.7	-1.6
Sodium dimethyldithio carbonate hydrate	4	0.2	29.3	28.6	-0.7
N-Benzylidene-benzylamine	4	0.1	28.6	28.3	-0.3
Melamine	4	0.2	27.7	25.9	-1.8
Anthracene	0.5	0.1	28.7	26.6	-2.1
Dicyandiamide	4	0.2	27.1	25.8	-1.3
Sulfanilic acid	4	0.2	26.2	24.1	-2.1
Sulfanilamide	4	0.2	26.2	23.8	-2.4
Urea	4	0.2	26.1	25.1	-1
Salicylic acid	3.5	0.1	27.0	24.3	-2.7
3,4,5-Trihydroxy-benzoic acid	3.5	0.1	27.0	24.2	-2.8
Ferric chloride	8	0.2	26.2	25.3	-0.9
Potassium ferricyanide	1	0.2	26.2	25.0	-1.2
Ascorbic acid	1	0.2	26.1	24.7	-1.4
Zincon	4	0.2	24.2	19.4	-4.8
Diisopropanolamine	4	0.2	24.2	19.5	-4.7
Adenosine triphosphate	4	0.2	24.2	23.1	-1.1

Guanidine	1	0.1	27.6	27.2	-0.4
Cyanuric acid	4	0.2	24.9	24.2	-0.7
Thiazol Yellow G	3	0.2	24.9	21.9	-3.0
Nicotinic acid	3	0.2	24.9	24.0	-0.9
Metanil Yellow	4	0.2	31.0	29.4	-1.6
Hardwood black liquor	1	0.1	31.0	30.9	-0.1
Softwood black liquor	2.5	0.2	28.5	26.5	-2.0
Methanesulfonic acid	4	0.2	25.2	24.9	-0.3
Metanilic acid	4	0.1	31.0	30.1	-0.9
Sulfamide	4	0.2	26.2	26.0	-0.2
3-Pyridine sulfonic acid	3	0.2	24.5	22.9	-1.6
Benzofuroxan	4	0.1	31.0	29.9	-1.1

t-Butyl hydroperoxide	5	0.1	24.2	23.1	-1.1
Pyruvic acid	4	0.2	24.7	24.3	-0.4
Imidazole	4	0.2	24.7	22.0	-2.7
N-Acetylcytosine	4	0.2	28.6	28.3	-0.3
Phenol	1	0.2	26.1	25.0	-1.1

It is to be understood that the above described embodiments are illustrative only and that modification throughout may occur to one skilled in the art. For example, a person of skill in the art will recognize that the mediators of the invention also include mediators which are functionally equivalent to the mediators specifically recited herein, such equivalents having minor structural variations such as the addition of a methyl or ethyl substituent or the formation of a methyl ester from a carboxylic acid. Accordingly, this invention is not to be regarded as limited to the embodiments disclosed herein.